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Nucleic Acids Research, 1998, Vol. 26, No. 7 **1707–1712**

Escherichia coli RNA and DNA polymerase bypass of dihydrouracil: mutagenic potential via transcription and replication

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Received December 3, 1997; Revised and Accepted February 4, 1998

ABSTRACT

Dihydrouracil (DHU) is a DNA base damage product produced in significant amounts by ionizing radiation damage to cytosine under anoxic conditions. DHU represents a model for pyrimidine base damage (ring saturation products) of the type recognized and repaired by *Escherichia coli* endonuclease III and its homologs in other species. We have built this lesion into synthetic oligonucleotides, with DHU placed at a single location downstream from an *E.coli* RNA polymerase promoter. This construct was used to determine the effect of DHU when encountered on a DNA template strand by either *E.coli* RNA or DNA polymerase (Klenow fragment). Single round transcription experiments or primer extension-type replication experiments were conducted in order to make a direct comparison between RNA and DNA polymerases with DHU placed within the same sequence context. Both DNA and RNA polymerase efficiently bypass DHU and insert adenine opposite this lesion. These results suggest that DHU is mutagenic with respect to both replication and transcription and have implications for DNA repair as well the routes leading to generation of mutant proteins in dividing and non-dividing cells.

INTRODUCTION

Cellular DNA is unstable and undergoes continuous chemical modifications from both exogenous and endogenous agents (1–5). Unrepaired DNA lesions can interfere with the DNA replication and RNA transcription machinery resulting in cell death, mutation and, in some cases, neoplastic transformation (1,6). 5,6-Dihydrouracil (DHU) is a pyrimidine ring saturation product generated by ionizing radiation under anoxic conditions (7). It is formed through deamination of cytosine and addition of hydrogen atoms at the C5 and C6 positions. Features of DHU which make it an attractive model for studies of DNA damage and repair include its recognition by *Escherichia coli* endonuclease III (endo III) and its homologs in other species as well as the ability to introduce DHU via chemical oligonucleotide synthesis into any DNA sequence context for *in vitro* or *in vivo* studies (7–9). Beyond recognition and removal by endo III and several eukaryotic endo III homologs little is known concerning the repair of DHU *in vivo*.

The majority of previous studies on the biological effects of unrepaired DNA damage have focused on the abilities of various lesions to either block or be bypassed by DNA polymerases (10–24). Such studies have allowed predictions concerning the extent to which a particular DNA damage may be viewed as a toxic (DNA polymerase-blocking) or mutagenic (DNA polymerase-bypass and miscoding) lesion. However, it has also become apparent that the interaction of DNA damage with RNA polymerases is important for determining whether a lesion is subject to transcription-coupled repair (TCR) as well as how it may directly affect various aspects of gene expression (25–30). Thus it is important to determine how a particular type of DNA damage interacts with both the replication and transcription machinery in order to understand the potential biological effects of such damage.

Previous studies in this laboratory have focused on the abilities of various types of DNA damage to block or be bypassed by single subunit phage RNA polymerases (31–35). These studies have served as useful simple models to predict: (i) what the potential effects of various classes of DNA damage might be on other types of RNA polymerase; (ii) whether or not such damage is likely to be subject to TCR; (iii) the mutagenic potential of such damage at the level of transcription. We now extend these studies to a more complex multisubunit RNA polymerase and make direct comparisons with DNA polymerase from that species.

In this report we have generated a duplex DNA template containing a single DHU lesion in the transcribed strand downstream from an *E.coli* RNA polymerase promoter. This construct was used to directly compare the effects of DHU on *E.coli* RNA and DNA polymerase when the template was utilized for *in vitro* transcription and replication experiments. Within the sequence context examined we find that both RNA and DNA polymerase efficiently bypass DHU and insert adenine opposite this lesion. These results indicate that DHU is mutagenic with respect to both replication and transcription and have several important implications for repair of this lesion as well as the routes leading to generation of mutant proteins in both dividing and non-dividing cells.

MATERIALS AND METHODS

Materials

The DHU dimethoxytrityl-blocked phosphoramidite building block was synthesized by Glenn Research (Sterling, VA). Oligonucleotides were synthesized by the Emory University Microchemical Facility or Life Technologies Inc. and purified by polyacrylamide

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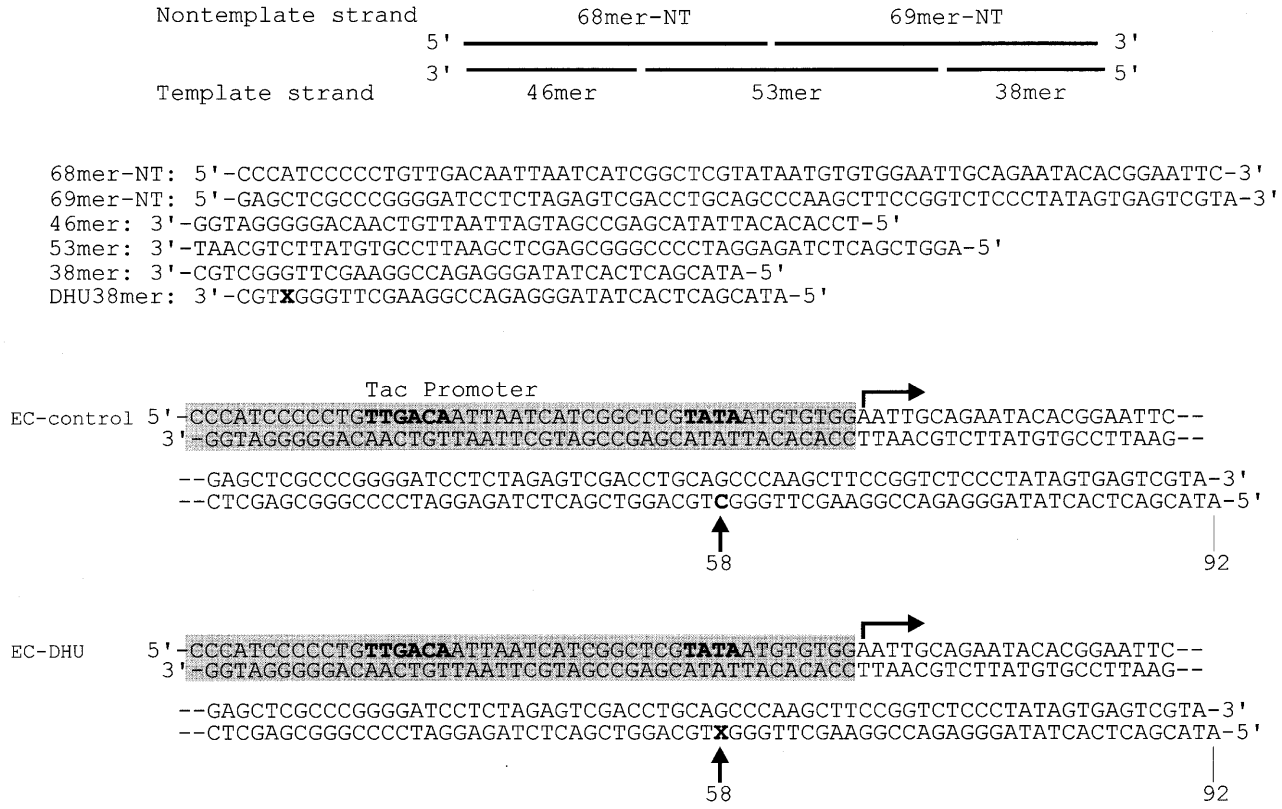

Figure 1. *Escherichia coli* RNA polymerase transcription templates and oligonucleotides from which these templates were synthesized (Materials and Methods). EC-control contains C (vertical arrow) and EC-DHU contains DHU (position X, vertical arrow) on the template strand 58 nt downsteam of the transcription start site (horizontal arrow). Shaded blocks indicate the *E.coli* RNA polymerase *tac* promoter.

gel electrophoresis (36). T4 polynucleotide kinase was obtained from New England Biolabs. T4 DNA ligase and *E.coli* DNA polymerase (Klenow fragment) were from Promega. *Taq* DNA polymerase was purchased from Fisher. Purified *E.coli* RNA polymerase was a gift from Dr Charles Turnbough (Birmingham, AL). Heparin and RNase inhibitor were purchased from Sigma. The Sequenase™ v.2.0 DNA Sequencing Kit was from US Biochemical; [α-^{35}S]dATP (sp. act. 1000 Ci/mmol) was from Amersham. The First Strand cDNA Synthesis Kit was purchased from Novagen. [α-^{32}P]CTP (sp. act. 3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham. HPLC-purified nucleoside triphosphates were from Pharmacia.

Construction of DNA templates for *E.coli* RNA polymerase transcription

Transcription templates (137 bp) for *E.coli* RNA polymerase experiments were generated by annealing and ligation of five separate component oligos as shown in Figure 1. To construct the control DNA template EC-control oligos 69mer, 46mer and 53mer (Fig. 1) were phosphorylated by T4 polynucleotide kinase in 25 µl kinase buffer containing ATP (0.2 mM final concentration) at 37° C for 18 h. Equal amounts (400 µM final concentration) of phosphorylated oligos 69mer-NT, 46mer and 53mer plus 68mer and 5′-^{32}P-labeled 38mer were annealed (10 µl final volume) by heating at 70°C for 10 min and then cooled to room temperature for 4 h. Ligation was carried out with 3 U T4 DNA ligase at 16°C for 18 h. After phenol/chloroform extraction template EC-control was purified from a 20% non-denaturing polyacrylamide gel (35). Template EC-DHU was constructed using a similar approach with DHU38mer replacing oligo 38mer (Fig. 1).

Construction of DNA templates for DNA replication experiments

To construct the DHU91mer for DNA replication experiments 4000 pmol 5′-^{32}P-labeled DHU38mer and 53mer (Fig. 1) were annealed and ligated on the complementary 69mer (Fig. 1). The conditions for annealing and ligation were as described above for the transcription experiments. A denaturing 20% polyacrylamide gel was used to separate and purify the DHU91mer from the 69mer (Fig. 5).

Single round transcription experiments

Single round transcription experiments were carried out by preincubation of 1 pmol DNA template with 10 mM DTT, 3 mM $MgCl_2$, 5 mM NaCl, 20 mM Tris–HCl, pH 7.9, 0.5 mM ATP, GTP and UTP, 3 U RNase inhibitor and 1 pmol *E.coli* RNA polymerase for 12 min at room temperature. After that heparin (250 µg/ml), CTP (10 µM) and 20 µCi [α-^{32}P]CTP (3000 Ci/mmol) were added to the preincubation mixture. Two microliter aliquots were removed and reactions were terminated by stop/loading buffer (9.8 M urea, 50 mM EDTA, 0.1% xylene cyanol) at increasing times. The ^{32}P-labeled transcripts were analyzed on a denaturing 15% polyacrylamide, 7 M urea gel and subjected to autoradiography and phosphorimager analysis.

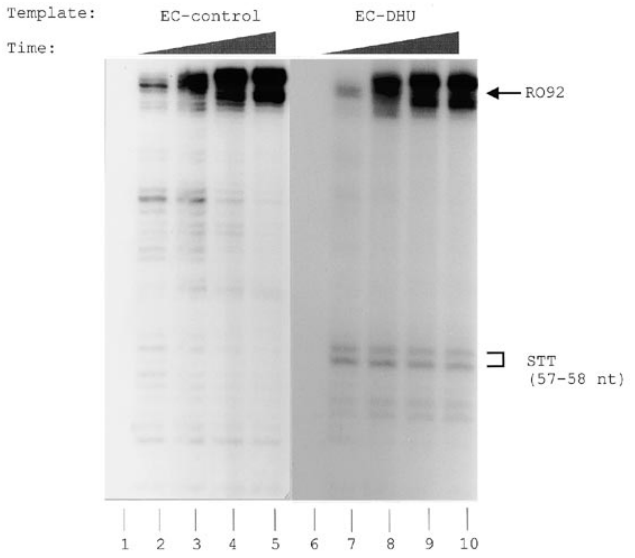

Figure 2. Single round transcription experiments with *E.coli* RNA polymerase on EC-control and EC-DHU templates. Region STT indicates stalled terminated transcripts (57 and 58 nt) and arrow RO indicates run-off transcripts (92 nt). Lanes 1–5 and 6–10 correspond to transcription products generated at 0, 30, 60, 120 and 240 s following the start of elongation.

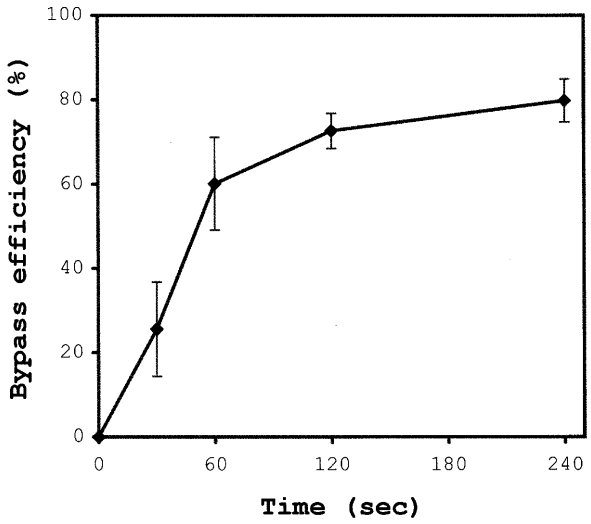

Figure 3. Bypass efficiency of *E.coli* RNA polymerase at sites of DHU on the transcribed strand. The relative amounts of stalled transcript STT and RO92 (bypass product) were calculated as described in Materials and Methods. Error bars represent standard deviations.

DNA replication experiments

DHU91mer and 5′-labeled oligo 18mer primer d(pTTGCAGAA-TACACGGAATT) were heated to 70°C for 5 min and incubated at 37°C for 5 min. After that 10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 7.5 mM DTT, 500 µM dNTPs and 5 U *E.coli* DNA polymerase (Klenow fragment) were added to the mixture. The incubation was carried out at 37°C for a total of 10 min. Reactions were terminated and end-labeled replication products were analyzed on denaturing polyacrylamide gels as described above for transcript analysis.

Transcription and replication product sequence analysis

RNA transcripts were generated under single round transcription conditions (described above) and cDNA synthesis was carried out using MMLV reverse transcriptase and 3′ antisense primer d(pATA-CGACTCACTATAGGGA) under conditions recommended by the supplier (Novagen). The cDNAs were PCR-amplified with the 3′ antisense primer and 5′ sense primer d(pTTGCAGAATACACG-GAATT) with *Taq* DNA polymerase. Sequence analysis was carried out with the Sequenase™ v.2.0 DNA Sequencing Kit (US Biochemical).

DNA replication products were subjected to 20% denaturing PAGE and the 5′-end-labeled products were isolated from the gel. PCR amplification and DNA sequence analysis of the 5′-end-labeled 91mer were carried out as described above.

Quantitation of DHU bypass efficiency of *E.coli* RNA polymerase

Quantitation of DHU bypass efficiency was based on the results of three separate single round transcription experiments. The relative amounts of stalled, terminated (STT) and run-off (RO) RNA transcripts at each time point were determined by phosphor-imager (Molecular Dynamics 445 SI) analysis of the denaturing polyacrylamide gel. For templates transcribed by *E.coli* RNA polymerase there are 27 cytosine residues in each full-length transcript and 16 cytosine residues in each stalled transcript. The percentage of STT was calculated as (STT) × (27/16)/[(STT) × (27/16) + (RO)] × 100 and the percentage of DHU bypass was calculated as (RO)/[(STT) × (27/16) + (RO)] × 100. This method of analysis is similar to bypass efficiency measurements made for other RNA polymerases (34).

RESULTS AND DISCUSSION

Generation of DNA templates containing a single DHU lesion for *in vitro* transcription and replication

To generate transcription templates EC-control and EC-DHU for the *E.coli* RNA polymerase experiments five different oligonucleotides were annealed, ligated and purified from a 20% non-denaturing gel (Materials and Methods). The resulting template EC-DHU contains a single DHU on the template strand 58 nt downstream of the start of transcription (Fig. 1). Transcription of EC-control by RNA polymerase should generate a full-length, run-off transcript 92 nt in length (RO92). DHU91mer (Fig. 5) was constructed for the primer extension-type replication experiments utilizing oligos DHU38mer, 53mer and 69mer (Materials and Methods, Fig. 5). DHU91mer contains a single DHU lesion located 57 nt from the 3′-end of the oligo.

Escherichia coli RNA polymerase bypasses DHU with high efficiency

In order to investigate the interaction of DHU and *E.coli* RNA polymerase elongation complex single round transcription experiments were carried out with templates EC-control and EC-DHU. Each template molecule is transcribed only once by a single molecule of RNA polymerase, which generates products reflecting a single, promoter-dependent transcription event (31). Time course analyses of single round transcription experiments

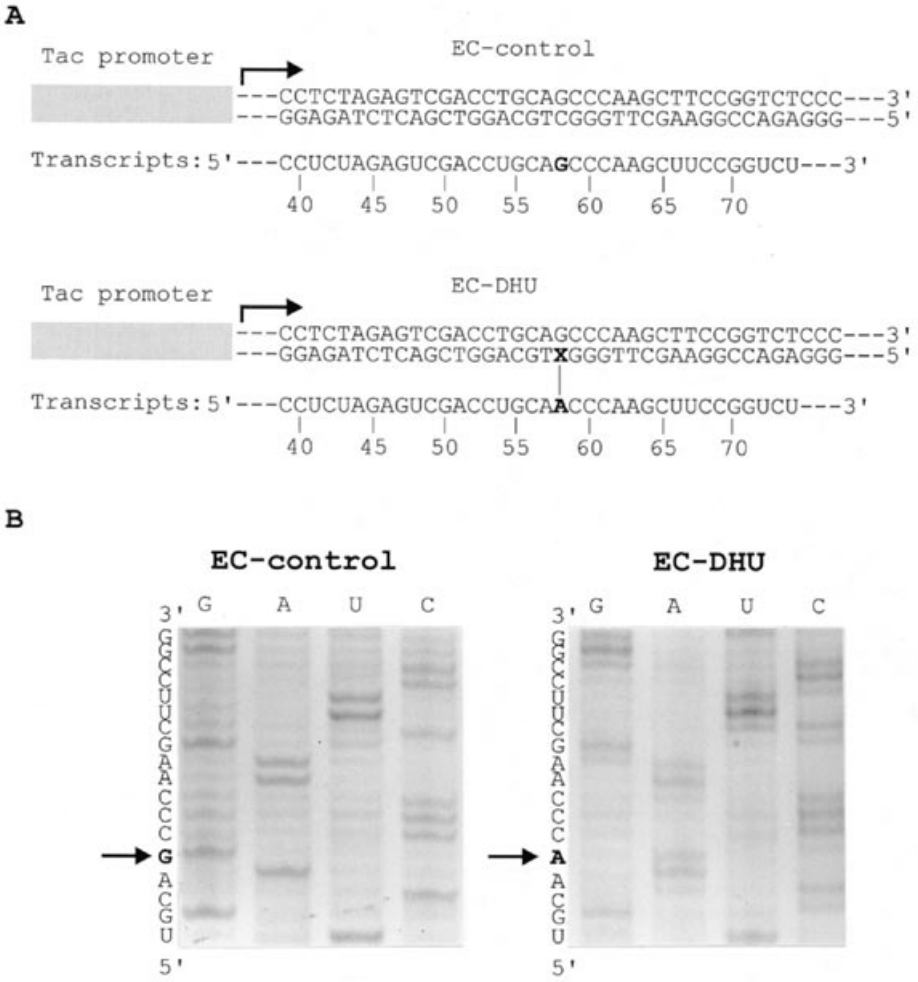

Figure 4. (**A**) Templates used in RNA sequence analysis. EC-control encodes a 92 nt full-length transcript and a portion of the predicted transcript sequence is shown. EC-DHU contains DHU on the template strand 58 nt from the transcription start site (horizontal arrow) and a portion of the deduced transcript sequence is shown. (**B**) Transcript sequence analysis. *Escherichia coli* RNA polymerase-generated transcripts from EC-control and EC-DHU templates were produced under single round transcription conditions and sequenced as described in Materials and Methods. The arrow indicates the nucleotide inserted at nucleotide position 58 from the 5′-end of the transcript, opposite cytosine (EC-control template) or DHU (EC-DHU template).

reveal potential polymerase temporary stalling events at specific template sites or other barriers to elongation, such as permanent arrest (31,34). As expected, transcription of template EC-control generated a 92 nt full-length run-off transcript (RO92), as shown in Figure 2 (lanes 1–5). At later time points 1 and 2 nt non-templated additions were observed, resulting in a slightly longer run-off transcript. Such non-templated additions are properties of several RNA polymerases, including *E.coli* RNA polymerase (35). Transcription of EC-DHU showed that *E.coli* RNA polymerase efficiently bypassed the DHU site and generated full-length run-off transcripts (Fig. 2). A minor percentage of stalled/terminated transcripts (STT57 and 58) were observed at the DHU site throughout the course of the single round transcription experiment and represented <20% of single round transcription events at 4 min. However, it is important to emphasize that the majority of RNA polymerase encounters with DHU resulted in efficient bypass and generation of a full-length transcript (RO92). The DHU bypassing efficiency of *E.coli* RNA polymerase is ~80% (Fig. 3), compared with almost 100% by phage RNA polymerases (34). The reason(s) for this difference is presently not known, but could be associated with differences in elongation rates, which are faster for phage RNA polymerases compared with *E.coli* RNA polymerase. A similar effect has also been observed between these polymerases with respect to their abilities to bypass template strand gaps (35).

Dihydrouracil causes mutagenic insertion for *E.coli* RNA polymerase

Since *E.coli* RNA polymerase efficiently bypassed DHU to generate run-off transcripts, it was of interest to determine which nucleotide(s) was inserted opposite DHU. The precursor base for DHU is cytosine, thus any base other than guanine inserted opposite DHU would be a base substitution mutation at the level of transcription. To determine the nature of the base inserted opposite DHU, RO92 transcripts from templates EC-control and EC-DHU were generated utilizing single round transcription conditions and isolated for sequence analysis (Materials and Methods). RT-PCR was carried out to generate cDNAs for DNA sequencing, which provided a direct read-out of the transcript sequence (Materials and Methods). Sequence analysis of RO92 produced from the EC-control template revealed the expected correctly templated nucleotide sequence (Fig. 4B). The transcript sequence deduced from RO92 generated from the EC-DHU template indicated that *E.coli* RNA polymerase inserts adenine opposite the DHU site, with no other bases inserted

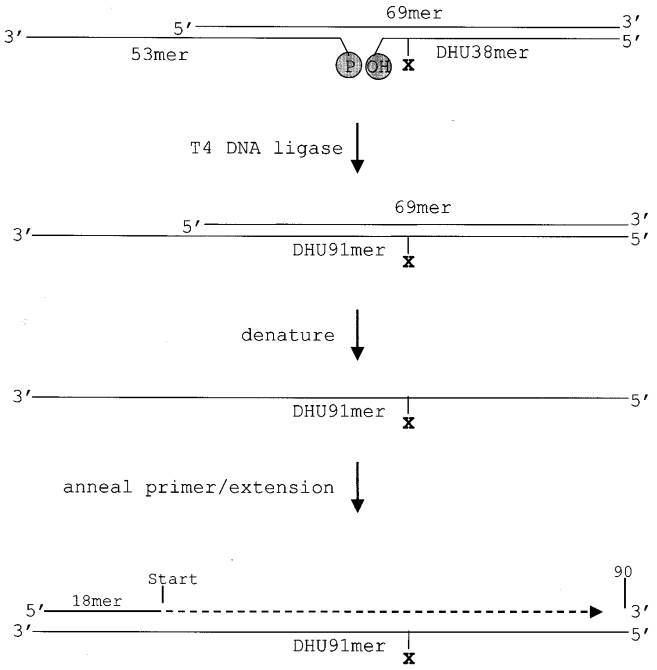

Figure 5. Construction of DNA synthesis template DHU91mer for replication studies. DHU38mer and oligo 53mer were linked together through the use of complementary oligo 69mer as a splinter. Following ligation DHU91mer was purified on a denaturing polyacrylamide gel. Primer annealing with oligo 18mer and extension with *E.coli* DNA polymerase (Klenow fragment) were as described in Materials and Methods. The start site for DNA synthesis (Start) corresponds to a position 21 nt downstream of the transcription start site using template EC-DHU as a reference (Fig. 1). The position of DHU (X) is 39 nt downstream (on the template) of the DNA synthesis start site and a full-length lesion bypass product has a predicted length of 90 nt.

at significant levels (Fig. 4B). Previous studies from our laboratory have indicated that both SP6 and T7 RNA polymerases insert primarily adenine, but also to a lesser extent guanine (34). *Escherichia coli* RNA polymerase differs from these phage polymerases in that no significant guanine insertion was observed. We conclude that DHU-mediated adenine insertion during transcription is a general property of prokaryotic RNA polymerases. It is reasonable to speculate that DHU is bypassed efficiently *in vivo* with the potential for producing mutant RNAs encoding mutant proteins.

Mutagenic bypass of DHU by *E.coli* DNA polymerase

We wished to determine whether DHU was capable of blocking or could be bypassed by *E.coli* DNA polymerase. For these studies the Klenow fragment of DNA polymerase I (containing polymerase and 3′→5′ exonuclease activities) was utilized. Primer extension-type replication experiments were carried out by employing an 18 nt DNA synthesis primer annealed to DHU91mer containing a sequence identical to the template strand used for transcription experiments (Fig. 5). Primer extension on the DHU91mer template resulted in a single product 90 nt in length (Fig. 6). No stalling at the site of DHU was observed and no shortened DNA synthesis products were detected. These results indicate that *E.coli* DNA polymerase efficiently bypassed DHU. The 90 nt product was purified from a denaturing polyacrylamide gel, PCR amplified and sequenced (Material and Methods). The sequencing results indicated that an adenine was inserted opposite DHU (Fig. 7). Since cytosine is the precursor of DHU, if such an event occurs *in vivo* this will result in a C→T transition mutation following replication. Because

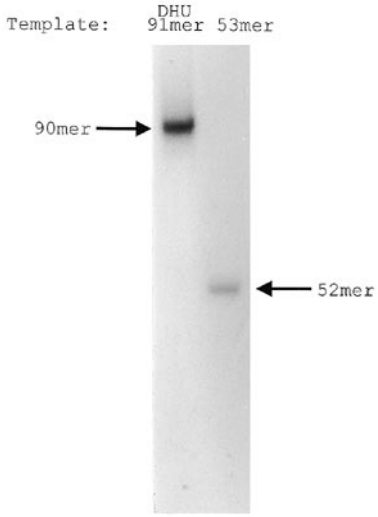

Figure 6. DNA replication experiments with Klenow fragment on templates DHU91mer and 53mer. DNA synthesis template DHU91mer contains a DHU lesion 57 nt from the 3′-end and was used in primer extension experiments as described in Materials and Methods. DNA synthesis template 53mer, identical to the 53mer used for construction of transcription templates (Fig. 1), was used for primer extension experiments to provide a size marker (52mer) to determine the length of potential shortened products due to polymerase blockage at the DHU site (Materials and Methods). It should be noted that the annealed primer is recessed 1 nt from the 3′-end of both templates, resulting in replication products 1 nt shorter than the template.

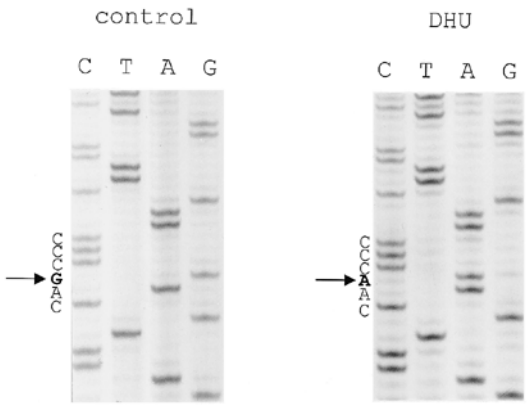

Figure 7. DNA sequence analysis of the 90 nt replication product generated from template DHU91mer. The 90 nt DNA product generated from template DHU91mer was subjected to PCR for DNA sequencing as described in Materials and Methods. The same primers were also used for PCR of template EC-control (undamaged template) to generate a 90 nt control DNA replication product. Arrows indicate the base insertion event opposite cytosine (control) or DHU.

the DNA polymerase used in this experiment contained 3′→5′ exonuclease proofreading activity, it is conceivable that bases (e.g. guanine) other than adenine could be initially inserted and then removed and replaced by adenine.

Conclusions

To our knowledge this is the first report of a direct comparison between DNA and RNA polymerase events at a lesion placed within the same DNA sequence context using *in vitro* transcription and replication systems. Since *in vitro* DNA and RNA polymerase systems are good predictors for similar events *in vivo*, the results

presented here suggest that DHU is mutagenic at the levels of both replication and transcription. It should be noted, however, that the studies reported here have examined DHU placed within a single sequence context and it is conceivable that different contexts may affect the absolute bypass efficiency of RNA and DNA polymerases over this lesion. Furthermore, the inability of DHU to strongly arrest RNA polymerase suggests that this lesion may not be subject to TCR and may lead to generation of substantial quantities of mutant transcripts. Thus DNA base damage products such as DHU, if left unrepaired, may cause generation of mutant proteins in cells regardless of their growth status. Previous studies which have focused separately on the effects of DNA polymerases and RNA polymerases on other base damage products, such as uracil (from cytosine deamination or misincorporation during replication) and 8-oxoguanine, indicate that these lesions are also mutagenic for replication and transcription (18–20,31; Viswanathan and Doetsch, unpublished results).

In the context of transcriptional mutagenesis the ability of a particular DNA lesion to produce mutant transcripts will depend upon the lifetime of the damage on the template strand, which will be governed by whether or not such a lesion is subject to TCR and, in the absence of TCR, how accessible it is to the 'global repair' machinery that is not associated with transcription. Nevertheless, it can be expected that transcriptional mutagenesis may be responsible for generation of appreciable quantities of mutant proteins in non-dividing cells. This may, in turn, result in a physiological change leading to a switch to a growth state with the potential to cause mutagenesis at the level of DNA replication via a DNA polymerase miscoding event in one of the daughter strands at the site of the same unrepaired damage (31,34,37). We are currently investigating whether or not such events occur in *E.coli* strains with different DNA repair backgrounds.

ACKNOWLEDGEMENTS

We thank Dr Charles Turnbough for the gift of *E.coli* RNA polymerase. We also thank the members of the Doetsch laboratory for technical assistance and helpful discussions. This work was supported by research grant CA 73041 from the National Cancer Institute.

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